The TIM-1:TIM-4 Pathway Enhances Renal Ischemia-Reperfusion Injury

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ABSTRACT

CD4⁺ T cells contribute to the pathogenesis of ischemia-reperfusion injury, which is the primary cause of delayed graft failure after kidney transplantation. The TIM-1:TIM-4 pathway participates in the activation/differentiation of CD4⁺ T cells, suggesting that it may modulate ischemia-reperfusion injury. Here, we studied the role of TIM-1 in a murine uninephrectomized renal ischemia-reperfusion injury model. Blocking the TIM-1:TIM-4 pathway with an antagonistic monoclonal antibody protected renal function and diminished reperfusion injury resulting from 30 minutes of ischemia. Histologic examination showed significantly less evidence of renal damage as evidenced by diminished tubular necrosis, preservation of the brush border, fewer cast formations, and less tubular dilation. Blocking TIM-1 also reduced the number of apoptotic cells and diminished local inflammation within ischemic kidneys, the latter shown by decreased recruitment of macrophages, neutrophils, and CD4⁺ T cells and by reduced local production of proinflammatory cytokines. Furthermore, TIM-1 blockade significantly improved survival after ischemia-reperfusion injury. Taken together, these data suggest that the TIM-1:TIM-4 pathway enhances injury after renal ischemia-reperfusion injury and may be a therapeutic target.


Ischemia-reperfusion (I/R) injury is a major cause of acute renal failure in native kidneys and in renal allografts and is associated with a high rate of mortality in patients and enhanced rate of rejections in transplanted kidneys.¹–⁴ The pathogenetic mechanisms of ischemic renal failure involve multiple mediators, such as cytokines, reactive oxygen species (ROS), adhesion molecules/chemokines, activation of leukocytes and endothelial cells that lead to tubular injury, endothelial dysfunction, and inflammation.⁵–⁸ Using T cell–deficient mice and adoptive transfer of T cells, Rabb and coworkers⁹ recently implicated a crucial role of T cells in the pathogenesis of I/R injury in the kidney. Furthermore, T cell–depleting reagents and blockade of co-stimulatory pathways have been reported to be beneficial in protection against I/R injury.¹⁰–¹³ Subsequent studies investigated the contribution of a Th1 and Th2 cytokine milieu in renal I/R injury using STAT4 and STAT6 knockout mice, finding that a Th1 shift has a deleterious effect in the pathogenesis of I/R, whereas a Th2 shift seems to be protective.¹⁴

The T cell Ig mucin (TIM) family of genes encodes proteins that are expressed by T cells and contain an IgV-like and a mucin-like domain.¹⁵,¹⁶ The TIM family consists of eight genes in mouse (TIM-1 to −8) and three genes in human (TIM-1, TIM-3, and TIM-4). TIM-1 was first identified as hepatitis...
A virus cellular receptor 1 (HAVCR1) and later as kidney injury molecule (KIM-1).\textsuperscript{17–19} KIM-1 is not detectable in normal kidney tissues but is highly upregulated on dedifferentiated tubular epithelial cells after ischemic or toxic kidney injury.\textsuperscript{18,20} KIM-1 expression on renal cells has been shown to trigger phagocytosis of apoptotic cells.\textsuperscript{21,22} In addition, TIM-1 is expressed on activated CD4\textsuperscript{+} T cells and upon polarization predominately on Th2 cells.\textsuperscript{23} TIM-1 ligation in combination with the T-cell receptor provides a positive co-stimulatory signal, resulting in an enhancement of T-cell proliferation, cytokine production, and abrogation of tolerance.\textsuperscript{23,24} Using an antagonistic anti-TIM monoclonal antibody (mAb), RMT1-10,\textsuperscript{25} we were able to show that TIM-1 blockade prolongs allograft survival by downregulation of Th1 cells and promotion of Th2-mediated alloresponses.\textsuperscript{26} TIM-4, which is expressed in high amounts on F4/80 macrophages, is the ligand for TIM-1, and TIM-1:TIM-4 interactions modulate the Th1/Th2 cytokine balance.\textsuperscript{21,27} Moreover, TIM-1 can regulate macrophage activation and alter the co-stimulatory properties of these cells.\textsuperscript{28}

To date, the role of the TIM-1 pathway in renal I/R injury is not known. Given the expression of TIM-1 on T cells and the emerging role of T cells in the pathogenesis of I/R injury, we speculated that TIM-1 might function as a novel target for prevention of renal dysfunction after ischemic kidney injury. Using the blocking anti-TIM-1 monoclonal antibody RMT1-10 in a murine (C57BL/6) uninephrectomized renal I/R injury model, we show that targeting the interaction of TIM-1:TIM-4 protects renal function and attenuates both the number of apoptotic cells and local inflammation within the ischemic kidney, resulting in significantly less histologic evidence of acute tubular necrosis and better survival after I/R injury.

RESULTS

TIM-1 Is Expressed on Activated CD4\textsuperscript{+} T Cells after Ischemic Injury

We studied the function of the TIM-1:TIM-4 pathway in I/R injury using the blocking anti-TIM-1 mAb RMT1-10 in a murine renal I/R injury model. In uninephrectomized male C57BL/6 mice, the remaining kidneys were clamped for 30 minutes at 37°C, and the mice were treated with RMT1-10 mAb or equal volume of saline (control mice) as mentioned in the Concise Methods section. Sham-operated mice were unilaterally nephrectomized only (sham mice).

To determine whether the expression of TIM-1 is induced after ischemic injury, we stained splenocytes obtained from control mice before or 6 and 24 hours after reperfusion with antibodies to CD4 and the marker CD69, characterizing activation of T cells, in combination with anti-TIM-1. We found that expression of TIM-1 was upregulated on CD4\textsuperscript{+}CD69\textsuperscript{+} T cells at 6 (mean fluorescence intensity, 77 versus 47; \textit{n} = 4, \textit{P} < 0.05) and 24 hours (mean fluorescence intensity, 74 versus 47; \textit{n} = 4, \textit{P} < 0.05) compared with T cells obtained from naïve mice (\textit{n} = 4), suggesting that TIM-1 expression is induced after ischemic injury (Figure 1).

RMT1-10 Protects against Reperfusion Injury and Prevents Renal Dysfunction

Renal function was assessed by measuring the serum creatinine levels at 6 and 24 hours after reperfusion. Control mice showed a significant elevation of serum creatinine at 6 (threefold over sham) and 24 hours (sixfold over sham) after reperfusion. The levels of serum creatinine for the control and sham groups were 116 ± 2 versus 35 ± 5 and 200 ± 3 versus 31 ± 1 µmol/L at 6 and 24 hours after reperfusion, respectively. However, RMT1-10 treatment protected renal function as shown by lower serum creatinine levels compared with control mice at 6 (81 ± 11 versus 116 ± 2 µmol/L, \textit{n} = 5, \textit{P} < 0.03) and 24 hours (163 ± 11 versus 200 ± 3 µmol/L, \textit{n} = 6, \textit{P} < 0.02) after reperfusion (Figure 2A). Aspartate aminotransferase (AST), which is not only present in liver cells but also in proximal tubular cells, is regarded as a nonspecific marker of extensive cellular disruption and/or necrosis and has been shown to be released during reperfusion after renal I/R injury.\textsuperscript{29,30} Therefore, we measured AST serum levels at the mentioned time points and used them as an indicator of reperfusion injury. In comparison with sham-operated mice, renal I/R led to a signif-
were significantly lower at both time points (6 hours: 81 vs. 116 serum creatinine levels in RMT1-10–treated animals (black bars) versus standard control mice (gray bars). Interestingly, serum AST levels, which are an indicator of cell integrity, were significantly improved in the RMT1-10 treatment group. At 6 hours, the mean serum AST level was 94 U/L in control mice versus 35 U/L in RMT1-10–treated mice. At 24 hours, the serum AST levels were 35 U/L in control mice versus 1709 U/L in RMT1-10–treated mice. This difference was statistically significant (P < 0.05).

Figure 2. RMT1-10 prevents renal dysfunction and protects against reperfusion injury. (A) Control mice (gray bars) show a dramatic elevation of serum creatinine levels at 6 (116 ± 2 versus 35 ± 5 μmol/L) and 24 hours (200 ± 3 versus 31 ± 1 μmol/L) after reperfusion over sham-operated animals (white bars), whereas serum creatinine levels in RMT1-10–treated animals (black bars) were significantly lower at both time points (6 hours: 81 ± 11 versus 116 ± 2 μmol/L, n = 5, P < 0.03; 24 hours: 163 ± 11 versus 200 ± 3 μmol/L, n = 6, P < 0.02). (B) Reperfusion injury, as evidenced by serum AST levels, was significantly improved in the RMT1-10–treated group compared with control animals (6 hours: 709 ± 94 U/L versus 1709 ± 290 U/L, n = 5, P < 0.01; 24 hours: 156 ± 35 U/L versus 421 ± 35 U/L, n = 6, P < 0.01). Serum creatinine and AST levels are expressed as the mean ± SEM of three independent experiments (at least five mice per group and time point). *P = 0.01 to 0.05, Significant; **P = 0.001 to 0.01, Very significant.

RMT1-10 Markedly Reduces Histologic Features of ATN
In control mice, parallel to the deterioration of functional parameters, histologic evaluation showed an early stage of acute tubular necrosis (ATN), evidenced by widespread tubular necrosis, loss of the brush border, cast formation, and tubular dilation in the outer medulla as early as 6 hours, whereas no evidence of ATN was seen in nonischemic kidneys of sham-operated animals. At 6 hours, the semiquantitative score for tubular injury in mice undergoing renal I/R was 3.4 ± 0.2 (n = 5). The pathologic features of acute tubular necrosis worsened at 24 hours after I/R with massive amounts of hyaline and granular casts in control animals (semiquantitative score for ATN, 3.7 ± 0.3; n = 5). Correlation of the functional data seen in mice treated with RMT1-10 renal sections obtained from this group showed a marked reduction of the histologic features of ATN in comparison to undergoing renal I/R alone at 6 (semiquantitative score for ATN, 2.2 ± 0.3 versus 3.4 ± 0.2; n = 5, P = 0.005) and 24 hours (semiquantitative score for ATN, 2.6 ± 0.2 versus 3.7 ± 0.3; n = 6, P = 0.01) after I/R (Figure 3, A and B).

Figure 3. RMT1-10 markedly reduces histologic features of ATN. Acute tubular injury (tubular necrosis, loss of the brush border, cast formation, and tubular dilation) in the outer medulla was evaluated in a semiquantitative manner in periodic acid-Schiff-stained kidney sections (0 = normal kidney; 1 = 1 to 25%; 2 = 25 to 50%; 3 = 50 to 75%, and 4 = 75 to 100% ATN). (A) The injury was worse in control animals (gray bars) compared with sham-operated mice (white bars) (6 hours: score 3.4 ± 0.2 vs. 0.15 ± 0.05, n = 5, P < 0.0001; 24 hours: score 3.7 ± 0.3 versus 0.15 ± 0.04, n = 6, P < 0.0001). However, RMT1-10 treatment (black bars) significantly reduced the ATN score after 6 and 24 hours of reperfusion (6 hours: score 2.2 ± 0.3 versus 3.4 ± 0.2, n = 5, P = 0.005; 24 hours: 2.6 ± 0.2 versus 3.7 ± 0.3, n = 6, P = 0.01). ATN score is shown as the mean ± SEM (at least five mice per group and time point). (B) Representative periodic acid-Schiff-stained sections of outer medulla from sham, control, and RMT1-10–treated animals (original magnification, ×200). *P = 0.01 to 0.05, Significant; **P = 0.001 to 0.01, Very significant.

RMT1-10 Significantly Prolongs Survival after I/R Injury
To assess the effect of RMT1-10 on survival after I/R injury, we monitored the viability of the mice in each group. In keeping with the preserved renal function and histology seen in mice treated with RMT1-10, blockade of the TIM-1 signal markedly prolonged survival compared with control mice (mean survival time, 24 versus 2 days; P < 0.005, n = 10), and 50% of the RMT1-10–treated mice survived indefinitely in contrast to none in the control group. As expected, a 100% survival rate was noted in sham-operated animals, suggesting that the surgical procedure was not a cause of death in this model. However, serial treatment with RMT1-10 (days 0, 2, 4, and 6) seemed to be more protective against I/R injury as a single dose on day of clamping, although the difference did not reach statistical significance (Figure 4).

Diminished T-Cell Activation in RMT1-10–Treated Animals after I/R Injury
In diseases involving T-cell trafficking into affected organs, upregulation of activation markers on T cells has been observed. Therefore, we measured the percentage of CD4+ T cells expressing the...
RMT1-10 Decreases Infiltration of Neutrophils, Macrophages, and CD4+ T Cells in Ischemic Kidneys

Quantification of infiltrating Ly-6B<sup>+</sup> neutrophils, F4/80<sup>+</sup> macrophages, and CD4<sup>+</sup> T cells into renal tissues by immuno-fluorescence staining showed that there was only a minimal number of all three cell types in nonischemic kidneys obtained from sham-operated mice. However, infiltrating Ly-6B<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages were observed in the outer medulla in mice subjected to I/R of the kidney as early as 6 hours and increased at 24 hours. Disruption of the TIM-1 signal by RMT1-10 significantly decreased the number of infiltrating neutrophils and macrophages compared with control mice at 6 (median of 13 versus 20.5 infiltrating cells per 15 high-powered fields [HPFs], n = 5, P < 0.001; median of 15 versus 18.5 infiltrating cells per 15 HPFs, n = 5, P < 0.05, respectively) and 24 hours (median of 16.5 versus 25 infiltrating cells per 15 HPFs, n = 6, P < 0.001; median of 18 versus

**Figure 4.** RMT1-10 significantly prolongs survival after I/R injury. The survival rates of sham, control, and RMT1-10–treated animals were compared by monitoring the viability of the mice in each group (n ≥ 6). A Kaplan-Meier plot shows a significantly better survival rate after treatment with RMT1-10. However, serial treatment with RMT1-10 (0.5 μg intraperitoneally on day 0 and 0.25 μg on days 2, 4, and 6) seems to be more effective than a single dose at time of I/R injury (0.5 μg intraperitoneally on day 0).

**Figure 5.** RMT1-10 diminishes T-cell activation. The percentage of activated CD4<sup>+</sup> T cells was assessed in freshly isolated leukocytes from spleens from sham (white box plots), control (gray box plots), and RMT1-10–treated (black box plots) animals by staining for the activation markers CD69 and CD25 and flow cytometric analysis. (A) The frequency of activated CD4<sup>+</sup> T cells expressing CD69 was significantly reduced in RMT1-10–treated mice compared with control mice (7.8 ± 0.7 vs. 11.1 ± 1%; n = 5, P < 0.05) and almost identical to that of sham–operated mice at the early time point at 6 hours, suggesting that RMT1-10 treatment abrogated T-cell activation. The same observation was true for the later time point at 24 hours (10.5 ± 0.6% in RMT1-10–treated mice vs. 14.9 ± 0.6% in control mice, n = 6, P < 0.05; Figure 5A), whereas the expression of the late activation marker CD25 was only slightly decreased at both time points (6 hours: 10.9 ± 0.6% in RMT1-10–treated mice vs. 13.9 ± 1.4% in control mice, n = 5, not significant; 24 hours: 10.8 ± 1.3% in RMT1-10–treated mice vs. 14.5 ± 1% in control mice, n = 6, not significant) (Figure 5B).
24.5 infiltrating cells per 15 HPFs, \( n = 6, P < 0.005 \), respectively. Although fewer infiltrating CD4\(^+\) T cells could be found in ischemic kidneys, they showed a similar kinetic as neutrophils and macrophages, with an increasing number over time and a maximal infiltration at 24 hours. The number of infiltrating CD4\(^+\) cells increased in control mice 2.5-fold over sham at 6 hours (median of 7.5 versus 3 infiltrating cells per 15 HPFs, \( n = 5, P < 0.005 \)) and 2.6-fold over sham at 24 hours (median of 16 versus 6 infiltrating cells per 15 HPFs, \( n = 6, P < 0.001 \)), whereas RMT1-10 reduced CD4\(^+\) T-cell infiltration compared with controls at both time points (median of 6 infiltrating cells per 15 HPFs, \( n = 5 \), not significant, and median of 8.5 infiltrating cells per 15 HPFs, \( n = 6, P < 0.005 \), respectively; Figure 6, A and B).

To evaluate whether circulating lymphocytes may be a source of infiltrating CD4\(^+\) T cells in the kidneys after I/R injury, we performed total CD4\(^+\) T-cell counts in the spleens of ischemic and nonischemic mice. Although CD4\(^+\) T-cell infiltration increased in the damaged kidneys during the reperfusion period, their number was significantly reduced in the spleens, suggesting that activated T cells infiltrate the kidney from lymphatic organs after I/R injury. The CD4\(^+\) T-cell number in the spleens was reduced by 51% at 6 hours and 22% at 24 hours in control mice compared with sham mice after reperfusion. In accordance with the diminished renal CD4\(^+\) T-cell infiltration seen in RMT1-10–treated mice, the absolute number of CD4\(^+\) T cells in the spleen was almost identical to the one observed in sham-operated mice and significantly en-
hanced compared with that seen in control mice. The number of CD8+ T cells did not differ between the three groups at any of the different time points (data not shown). Collectively, these data in combination with the reduced infiltrating CD4+ T cells in ischemic kidneys suggest that RMT1-10 may protect against I/R injury by reducing CD4+ T-cell activation in lymphoid organs and thereby delaying T-cell migration to the damaged kidneys.

**Diminished Cell Infiltration in Ischemic Kidneys is Associated with Decreased Cytokine Production**

Given the importance of Th1/Th2 cytokines in the pathogenesis of I/R injury and that TIM-1 is primarily expressed by Th2 cells, we reasoned RMT1-10 may prolong survival after I/R injury by tipping the Th1/Th2 balance toward a Th2 response. To explore this possibility, we assessed the level of Th1 (IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-5) cytokines after I/R injury in serum by flow cytometric analysis using Cytometric Bead Array (CBA) beads. Despite several reports showing increased IFN-γ serum levels in animals subjected to I/R injury,32 IFN-γ was not detectable in the serum in our model. The same was true for IL-2 and IL-4 serum levels (data not shown). Although to a low degree both the serum levels of TNF-α (6 hours: 26.4 ± 4.7 versus 8.5 ± 0.29 pg/ml, P < 0.05; 24 hours: 33.3 ± 4.7 versus 11.9 ± 1 pg/ml, P < 0.05) and IL-5 (6 hours: 29 ± 5.8 versus 4.3 ± 0.4 pg/ml, P < 0.05; 24 hours: 6.2 ± 1.3 versus 4.5 ± 0.5 pg/ml, not significant) increased in mice subjected to I/R compared with sham-operated mice. As expected, RMT1-10 treatment led to a reduction of TNF-α compared with control mice (6 hours: 26.44 ± 4.7 versus 17.3 ± 3.7 pg/ml, not significant; 24 hours: 33.3 ± 4.7 versus 24.5 ± 4.5 pg/ml, P < 0.05). However, in contrary to our hypothesis, treatment with RMT1-10 reduced the production of the Th2 cytokine IL-5 slightly at 6 hours (26.44 ± 4.7 versus 22 ± 7 pg/ml, not significant) but not at 24 hours after I/R, probably reflecting a general reduced T-cell activation.

To assess the role of key T-cell and macrophage activation products locally at the site of injury, we analyzed the expression of the cytokines IFN-γ, IL-10, TNF-α, and IL-6 by real-time PCR in ischemic kidneys. As expected, sham-operated kidneys did not express altered cytokine levels in comparison to naive kidneys. However, kidneys subjected to I/R alone showed a marked upregulation of TNF-α (at 6 hours: 1.0- versus 8.2-fold, P < 0.0001; at 24 hours: 0.9- versus 7.4-fold, not significant) and IL-6 (at 6 hours: 2- versus 16-fold, P < 0.0001; at 24 hours: 0.4- versus 9.5-fold, P < 0.0001) at both time points, whereas IFN-γ (at 6 hours: 0.9- versus 0.5-fold, not significant; at 24 hours: 1.0- versus 5.5-fold, P < 0.05) and IL-10 (at 6 hours: 4.1- versus 7.7-fold, not significant; at 24 hours: 1.9- versus 8.1-fold, P < 0.05) were only upregulated at 24 hours after I/R injury compared with sham animals. Consistent with the diminished T-cell infiltration in RMT1-10–treated animals, real-time PCR showed a reduced expression of the cytokines produced by activated T cells predominately at 24 hours (IFN-γ, 0.6- versus 5.5-fold, P < 0.05; IL-10, 3.6- versus 8.1-fold, P < 0.05), whereas the macrophage products TNF-α (at 6 hours: 4.4- versus 8.2-fold, P < 0.5; at 24 hours: 2.3- versus 7.4-fold, not significant) and IL-6 (at 6 hours: 13-versus 16-fold, not significant; at 24 hours: 4.6- versus 9.5-fold, P < 0.005) were reduced at both time points compared with control mice (Figure 7), confirming at a molecular level the observed difference in organ function mentioned above.

**RMT1-10 Reduces Apoptosis in Ischemic Kidneys**

I/R injury is associated with ischemia-induced apoptosis, which might contribute to renal dysfunction. Recently tubular epithelial cells expressing KIM-1 were shown to phagocytose apoptotic and necrotic cells in vivo.21,22 To evaluate the impact of RMT1-10 on the development of apoptosis in ischemic kidneys, we scored the number of TUNEL-positive cells by deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay and for cleaved caspase 3 intensity. In control mice, 89 ± 6 of TUNEL-positive cells were noted in outer medulla, whereas only a few TUNEL-positive cells were seen in non-ischemic kidneys of sham-operated animals (2 ± 0.4, P <
However, RMT1-10 markedly reduced the number of TUNEL-positive cells compared with control mice (51/89 versus 89/6, n = 5, P = 0.001). Consistently, the cleaved caspase 3 intensity, as assessed by a semiquantitative score, increased in control mice ninefold over sham (median score of 0.5 versus 4.5, n = 5, P < 0.005), whereas RMT1-10 significantly decreased cleaved caspase 3 intensity compared with controls (median score of 3, P = 0.0005; Figure 8, A and B). In summary, RMT1-10 attenuated the number of apoptotic cells in ischemic kidneys and may thereby limit the local injury/inflammation, leading to a decreased recruitment of neutrophils and macrophages and reduced activation of adaptive immune mechanisms.

RMT1-10 Has No Effect in RAG−/− Animals Lacking T Cells

Because TIM-1 has been shown to be expressed on activated T cells and on ischemic tubular epithelial cells (KIM-1), RMT1-10 might protect the kidney against ischemic injury by targeting either cell type. To discriminate between the two possibilities, we studied the effect of RMT1-10 after I/R injury in RAG−/− animals, which lack T cells. Despite several reports, we noted only a minimal, but statistically significant, prolonged survival rate in Rag−/− mice in comparison to wild-type mice when subjected to I/R injury (mean survival time, 4.5 versus 2 days, P < 0.01, n = 6). However, we were unable to show a protective effect of TIM-1 blockade in RAG−/− animals because the survival rate observed in RMT1-10–treated RAG−/− animals did not differ from that in control RAG−/− animals after I/R injury (Figure 9A), indicating that the target of RMT1-10 in our model is TIM-1 on T cells rather than KIM-1 on tubular epithelial cells. In keeping with the survival data, we were unable to identify marked changes in the expression of the Th1 cytokine IFN-γ (Figure 9C), and Th2 cytokine IL-10 (Figure 9D) in RMT1-10–treated and control RAG−/− animals at 24 hours. However, the expression of both cytokines was markedly reduced in ischemic kidneys of RAG−/− animals compared with those from wild-type animals, indicating that T cells are the source of these factors. Interestingly, the protection of TIM-1 blockade was restored after adoptive transfer of wild-type splenocytes into RAG−/− animals, suggesting that RMT1-10 might regulate the innate immune response via T cells (Figure 9B).

DISCUSSION

This is the first study to our knowledge establishing the in vivo function of the TIM-1:TIM-4 pathway after renal I/R injury. We clearly showed that targeting the TIM-1:TIM-4 pathway using the monoclonal antibody RMT1-10 protected renal function (decrease in serum creatinine) and diminished the reperfusion injury (decrease in serum AST) after 30 minutes of
Ischemic renal injury. RMT1-10–treated mice had significantly less acute tubular necrosis and diminished local inflammation characterized by a decreased recruitment of leukocytes (neutrophils, macrophages, and CD4+ T cells) and reduced local production of proinflammatory cytokines. In accordance with these findings, TIM-1 blockade significantly ameliorated survival rates after I/R injury. The better survival was associated with a reduced number of apoptotic cells within the ischemic kidney.

T cells constitute one of the primary arms of the adaptive immune response, and according to the classical immunological dogma, these cells were not suspected to play a role in ischemic injury. However, recent studies suggest a crucial role of T cells as important mediators in I/R injury. T cell–deficient or T cell–depleted mice showed marked protection against I/R injury. Furthermore, FY720 or the S1P1 agonist SEW2871, both agents known to prevent T-cell emigration from secondary lymphatic organs, protected kidney function after ischemic injury. Especially the CD4+ T cells were identified as the important T-cell subset that mediated renal I/R injury. We noted the accumulation of CD4+ T cells after reperfusion in mice subjected to ischemic injury as early as 6 hours. The recruitment of CD4+ T cells into the damaged kidneys was associated with a significant increase in the ratio of CD69-expressing activated CD4+ T cells and a decrease in the absolute number of CD4+ T cells in the spleens. This association suggests that circulating T cells are activated in a non–antigen-specific way and participate in the initiation of the inflammatory response after renal I/R injury. These data are in keeping with previously published data by Lai et al. showing that during the reperfusion period CD4+ T cells increased in the damaged interstitium of the cortical medullary junction, whereas they were reduced in the blood. However, treatment with RMT1-10 significantly reduced the percentage of activated T cells within the spleens and CD4+ T-cell infiltrates in the damaged kidneys, whereas the total number of CD4+ T cells was unaltered in the spleen, implicating that RMT1-10 alters activation and recruitment of CD4+ T cells to the site of injury.

The mechanism of T-cell infiltration during the early phase of I/R injury is unknown. The rapid movement suggests that it is mediated through an antigen-independent activation. Oxygen free radicals, RANTES, and proinflammatory cytokines including IFN-γ, IL-2, TNF-α, and IL-6 have been shown to recruit and activate T cells directly. We found a sustained upregulation of the cytokines IFN-γ, TNF-α, and IL-6 within ischemic kidneys, implicating that these cytokines are potential mediators in the pathogenesis after I/R injury in our model. However, once recruited and activated T cells can, in turn, produce TNF-α and IFN-γ themselves, suggesting that besides T-cell trafficking to the site of injury the function of these cytokines locally could be crucial for the pathogenesis after I/R injury. CD4+ T cells were reported to require IFN-γ to be able to generate injury, and IFN-γ has been shown to generate a number of detrimental changes, including disruption of cell-matrix adhesion, inducing cell shedding into the lumen, upregulation of adhesion molecules and selectins, and re-

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**Figure 9.** The protective effect of RMT1-10 after I/R injury is abrogated in the absence of T cells. The survival rates of sham, control, and RMT1-10–treated RAG−/− animals were compared by monitoring the viability of the mice in each group. (A) A Kaplan-Meier plot shows a similar survival rate in RAG−/− animals after treatment with RMT1-10 (0.5 μg intraperitoneally on day 0 and 0.25 μg on days 2, 4, and 6) compared with control animals (n = 6 mice/group). (B) However, treatment with RMT1-10 significantly prolongs survival in RAG−/− animals that received adoptive transfer of wild-type splenocytes 1 day before I/R injury was induced (d = 1). Real-time PCR showed a similar expression of IFN-γ (C) and IL-10 (D) within the ischemic kidneys in RMT1-10–treated versus nontreated RAG−/− animals compared to the marked reduction of both cytokines in wild-type (WT) animals after treatment with RMT1-10. Data were normalized to the expression of β-2-microglobulin gene expression and are shown as fold increase over the expression in healthy kidneys from naïve WT or RAG−/− mice. Data are presented as mean ± SEM (at least four mice per group).
recruitment/activation of further leukocytes. One of the striking effects after RMT1-10 treatment was the marked decrease in IFN-γ and TNF-α within the ischemic kidneys. Thus, blocking TIM-1 might abrogate the initiation of immune response after I/R by preventing early T-cell activation/recruitment within ischemic kidneys. However, the higher number of CD4⁺ T cells within ischemic kidneys at 24 hours compared with 6 hours suggest that T cells also mediate progression and/or maintenance of the inflammatory response after ischemic injury in our model. Furthermore, serial treatment with RMT1-10 proved to be more protective compared with a single dose, indicating that RMT1-10 reduces late T-cell responses after ischemic injury.

Another striking difference between RMT1-10–treated and control animals after I/R injury was a markedly reduced number of infiltrating F4/80⁺ macrophages and production of IL-6 in ischemic kidneys. This is consistent with recently published data from Uchida et al., showing that TIM-1 is critical for the T cell–macrophage cross-talk in that blocking the TIM-1:TIM-4 pathway decreases macrophage activation and recruitment within ischemic damaged livers. Because RMT1-10 diminished local production of proinflammatory cytokines, TIM-1 blockade might reduce macrophage recruitment and activation in inflammatory cascade by an indirect pathway. However, the expression of TIM-4 is highly restricted to antigen presenting cells and is expressed in high amounts on F4/80⁺ macrophages. Targeting the TIM-1:TIM-4 pathway has been shown to regulate macrophage activation, leading to alteration in the co-stimulatory properties of these cells. Thus, RMT1-10 might also block CD4⁺ T cell–macrophage interaction directly. Regardless of the pathway, prevention of macrophage recruitment/activation reduces local damage and may protect organ function.

Another aspect of our study was to examine the importance of Th1/Th2 immune response in I/R injury. Current data suggest that the Th1 immune response in I/R injury could be injurious, whereas the Th2 phenotype could be protective. Using RMT1-10, we were able to show that TIM-1 blockade prolongs allograft survival in a fully mismatched murine heart transplant model by downregulation of Th1-mediated and promotion of Th2-mediated alloreponses. Therefore, we reasoned that RMT1-10 might protect against I/R injury by modulating the Th1/Th2 cytokine balance. However, contrary to our expectations, systemic Th2 cytokine levels (IL-5 in serum) and local cytokine expression within ischemic kidneys (IL-10 mRNA) decreased in a similar manner as Th1 cytokine levels did, probably reflecting an overall decrease in T-cell activation. Thus, the beneficial effects of RMT1-10 after I/R injury are not related to a Th2 cytokine shift in our model.

Renal tubular cells (RTC) die by apoptosis or necrosis in experimental models of I/R injury, and it is well established that apoptosis contributes to the loss of renal tubular cells in ischemic kidney injury. Among others, exposure of RTCs to inflammatory cytokines or ATP depletion (which induces cytokine release from these cells) lead to an up-regulation of the expression of the receptors FasL (Fas ligand) and TNF receptor 1, and the cells become sensitized to Fas- and TNF-α–mediated apoptosis. Thus, provision of the ligands Fas and TNF-α during I/R injury may contribute to apoptosis in acute renal failure. Neutralization of TNF-α has shown to inhibit ischemia-induced RTC apoptosis. RMT1-10–treated ischemic kidneys showed a reduced expression of TNF-α accompanied by a diminished frequency of TUNEL-positive cells and cleavage of caspase 3, suggesting that TIM-1 blockade might downregulate apoptotic pathways. On the other hand, it has been shown that TIM-1 and TIM-4 can enhance phosphatidylserine-dependent clearance of apoptotic cells. However, contrary to these studies, we observed a reduction of apoptotic cells within ischemic kidneys after blocking of the TIM-1 signal, indicating that RMT1-10 might limit the number of apoptotic cells by diminishing the local injury/inflammation (e.g., TNF-α) rather than initiating engulfment of apoptotic cells.

Mice lacking T or B cells have been shown to have less tissue damage on I/R injury in some reports, whereas other studies showed similar severity of ischemic injury in RAG⁻/⁻ compared with wild-type mice, suggesting an increase in innate immune responses (e.g., NK cell activity) in RAG⁻/⁻ mice as the compensatory mechanism for loss of T and B cells. We observed a minimal, although statistically significant, prolongation of survival in RAG⁻/⁻ compared with wild-type animals after I/R injury. Consistently with our hypothesis that RMT1-10 blocks T-cell activation, anti-TIM-1 blockade had no effect in RAG⁻/⁻ animals lacking T cells. However, its protective effect was restored after the adoptive transfer of splenocytes containing T cells, suggesting that RMT1-10 might regulate the innate immune response in RAG⁻/⁻ mice through T cells.

In summary, renal I/R injury triggers TIM-1 signaling to activate T cells, which in turn have a downstream effect on later inflammation and organ dysfunction. TIM-1 blockade protects renal function by inhibiting T-cell activation, leading to decreased recruitment of macrophages and neutrophils and attenuation of apoptosis. This study provides evidence for a novel mechanism by which TIM-1 signaling affects innate immunity-driven inflammatory responses during the course of renal I/R injury. Disruption of TIM-1:TIM-4 may serve as a novel target for the therapy after renal I/R injury.

**CONCISE METHODS**

**Animals**

Male C57BL/6 and B6.129S7-Rag1tm1Mom/J (RAG⁻/⁻) mice were purchased from Charles River (Sulzfeld, Germany). All mice were used at 9 to 12 weeks of age. Mice were housed, and all procedures were performed in accordance with institutional guidelines.

**Uninephrectomized Renal I/R Injury Model**

Mice were anesthetized by inhalation of isoflurane, and a right flank incision was made. The right renal pedicle was ligated, the right kidney was removed, and the wound was sutured with 4-0 silk. The mice...
were allowed to recover from anesthesia and surgery for 3 days. Then, the mice again were anesthetized as mentioned above and placed on a heating pad set to maintain the temperature of the mice at 37°C. A left flank incision was made, and the left renal pedicle was occluded with a nontraumatic microvascular clamp. The mice were kept on the heating pad during the whole procedure. Thirty minutes after placement of the clamp, the organ was allowed to reperfuse by removal of the clamp, and the wound was sutured with 4-0 silk. The animals were allowed to recover from anesthesia and surgery with free access to food and water. Sham operation was performed in a similar manner, except for clamping of the renal vessels. Animals were killed 6 and 24 hours after I/R.

Antibodies and In Vivo Treatment Protocol
Recipient mice were injected intraperitoneal with the monoclonal antibody RMT1-10 or 0.9% sodium chloride vehicle on day 0 (0.5 μg) and on days 2, 4, and 6 (0.25 μg) after reperfusion. RMT1-10 was manufactured by Bioexpress Cell Culture.

Renal Function
Blood samples were taken from the retro-orbital vein at each time point to evaluate renal function by measurement of serum creatinine by an autoanalyzer (Beckman Analyzer; Beckman Instruments, Munich, Germany). In addition, serum AST levels were studied as an indicator of reperfusion injury.

Tissue Preparation and Histology
Kidneys were removed at 6 and 24 hours for pathologic examination and quantification of gene expression. One third of the kidney was placed in optimum cutting temperature (OCT) compound for immunohistochemistry. One third was snap-frozen for real-time PCR.

The remaining third was fixed in 4% paraformaldehyde followed by embedding in paraffin and staining with periodic acid-Schiff reagent. The severity of tubular injury in kidney sections was performed in a blinded fashion. Tubular necrosis was evaluated in a semiquantitative manner by determining the percentage of tubules in the outer medulla in which epithelial necrosis, loss of the brush border, cast formation, and tubular dilation was observed. A five-point scale was used: 0, normal kidney; 1: 1 to 25%; 2: 25 to 50%; 3: 50 to 75%; and 4, 75 to 100% tubular necrosis.

Immunofluorescence
Immunofluorescence was performed on cryosections (6 μm) using the following primary antibodies: rat anti-mouse CD4 (clone H129.19; BD Biosciences) and rat anti-mouse F4/80 (clone A3–1; AbD Serotec). The following primary antibodies were used on formalin-fixed paraffin sections (2 μm): rat anti-mouse Ly-6B (clone 7/4; AbD Serotec) and rabbit anti-cleaved caspase-3 (Asp175, cell signaling). For indirect immunofluorescence, nonspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories) for 30 minutes in room temperature. Thereafter, sections were incubated with the primary antibody for 1 hour. All incubations were performed in a humid chamber at room temperature. For fluorescence visualization of bound primary antibodies, sections were further incubated with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 hour in the dark. For negative controls, the staining procedure was performed as described without the primary antibodies. Specimens were analyzed using a Zeiss Axioplan-2 imaging microscope with the digital image-processing program AxioVision 4.3 (Zeiss, Jena, Germany). Semiquantitative analysis of cell infiltration was done by counting the number of positively labeled cells in 15 nonoverlapping HPFs. Analysis of cleaved caspase 3 expression was done by using the following arbitrary units (0 to 5): 0, absent; 1, very weak; 2, weak; 3, moderate; 4, strong; and 5, very strong expression. The investigator performing these immunohistochemical analyses had no knowledge of the treatment group assignment.

TUNEL-Diaminobenzidine Assay
For the TUNEL assay, 2-μm sections of 4% paraformaldehyde-fixed paraffin-embedded tissues were deparaffinized, treated with the terminal deoxynucleotidyl transferase enzyme, and incubated in a humidified chamber at 37°C for 1 hour. After washing, the tissue was treated with FITC-labeled anti-digoxigenin, incubated for 60 minutes, and washed. Negative controls were prepared under the same conditions, with the omission of the terminal deoxynucleotidyl transferase enzyme. TUNEL-positive cell numbers were counted in 20 nonoverlapping view fields in the outer medulla per specimen (magnification, ×400) without knowledge of the animal assignment.

RNA Extraction and Real-Time Quantitative PCR
Total RNA was extracted using RNeasy mini columns with an on-column DNase-digest (Qiagen, Hilden, Germany). Concentration of the RNA was determined, and 2 μg of total RNA was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). Quantitative PCR was performed on a SDS 7700 system (Applied Biosystems, Darmstadt, Germany) with FastStart taq Polymerase (Roche Diagnostics) in combination with SYBR-Green chemistry (Invitrogen). Gene-specific oligonucleotides for IFN-γ (QT01038821), IL-10 (QT00106169), IL-6 (QT00098875), and TNF-α (QT00104006) were obtained from Qia-gen (QuantiTect Primer Assay) with the corresponding ordering numbers listed in brackets. PCR results were normalized to the expression of β-2-microglobulin, and data were analyzed using Q-gene software. Data are shown as fold change over the expression of the gene of interest in apparently healthy kidneys from naïve male C57BL/6 mice.

FACS Analysis
Spleens were harvested from naïve mice, as well as 6 and 24 hours after I/R, and single cell suspensions were prepared. The number of CD4+ and CD8+ T cells and the frequency of activated CD4+ and CD8+ T cells expressing TIM-1, CD25, or CD69 were measured by flow cytometry analysis (FACSCalibur) and analyzed using FlowJo software (Flow cytometry Analysis Software; Tree Star). All antibodies were obtained from BD Pharrmingen with the exception of anti-TIM-1, which was obtained from ebioscience.
Cytokine Assay
Serum levels of IFN-γ, TNF-α, IL-2, IL-4, and IL-5 at 6 and 24 hours were detected using the mouse Th1/Th2 cytokine CBA kit from BD Biosciences. Fifty microliters of serum sample was mixed with 50 μl of the mixed capture beads and 50 μl of the mouse Th1/Th2 PE detection reagent. The tubes were incubated at room temperature for 2 hours in the dark, followed by a wash step. The samples were resuspended in 400 μl of wash buffer before acquisition on the FACScan. The data were analyzed using the CBA software. Standard curves were generated for each cytokine using the mixed bead standard provided in the kit, and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve. Means and SD were determined using data from individual animals.

Adoptive Cell Transfer
RAG−/− mice were intravenously injected with total red blood cell-depleted splenocytes (20 × 10⁶ cells/mouse) from wild-type animals 1 day before I/R injury was induced (d−1).

Statistical Analysis
Data are expressed as mean ± SEM; t test was used for comparison of means between experimental groups. Differences were considered to be significant at P < 0.05. Analysis of data was performed using GraphPad PRISM software (GraphPad Software, La Jolla, CA).

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DISCLOSURES
None.

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